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Endogenous carcinogens, cancer-causing agents formed in mammalian cells, have been poorly investigated. Our group has hypothesized and furnished evidence that oxidized metabolites of catechol estrogens (CE) initiate breast cancer by damaging DNA in breast cells. These active metabolites, the quinones of CE (CE-Q), bind to DNA and form two types of adducts: depurinating adducts that generate apurinic sites, and stable adducts that remain linked to DNA. It is logical to speculate that for the progression from DNA damage to mutation, the repair mechanisms within cells must sometimes fail. The main objective of these experiments is to demonstrate CE-Q-induced DNA damage and to shed light on the mechanism of faulty repair. First I have determined the level of DNA damage by CE and the cell type which underwent this damage by use of an unscheduled DNA synthesis assay in ACI rat mammary gland organ culture. Then, I have tried to design molecular biological techniques that display DNA damage. The most successful technique uses a series of radiolabeled single-strand oligonucleotides which contain a single site that can undergo CE-induced DNA damage creating strand breaks that are visualized on a polyacrylamide gel.

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Kimberly A. Chapman

PI - Signature Date

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INTRODUCTION

One in every nine women develops breast cancer in her lifetime. Our laboratory and others implicate estrogens as one of the major reasons for this scary statistic. Evidence from our laboratory and others indicates that estrogens can act as complete endogenous carcinogens in initiating and promoting the development of cancer. Historically, the role of estrogens has been related to stimulation of proliferation by receptor-mediated processes (1-3). In more recent years, estrogens have also been shown to act as endogenous DNA-damaging agents by generating electrophilic species that can covalently bind DNA and initiate cancer (4,5). In other words, oxidized estrogen metabolites can directly bind DNA. Furthermore, I hypothesize that the results of this binding may cause failure to repair or mis-repair of the DNA damage leading to mutation and possibly to cancer.

In more scientific terms, 17β-estradiol (E_2) and estrone (E_1), which are continuously interconverted, can be oxidized to their catechol forms (CE) at the 2 or 4 position by cytochrome P450s and then oxidized by cytochrome P450s or peroxidases to quinone forms (CE-Q) (6). These CE-Q damage DNA by directly binding to the DNA and forming stable adducts, which remain in the DNA, or depurinating adducts, which are lost when the bond between the sugar and nucleobase is broken (4,5,7,8). Mammalian cells care usually protected from this damage by catechol-O-methyltransferase (COMT) and glutathione S- transferases which inactivate the CE and CE-Q (5,6,9).

In vitro and *in vivo* assays indicate that these CE-Q bind to the N7 of guanine or the N3 of adenine to form depurinating adducts that lead to apurinic sites in the DNA. CE-Q-initiated stable adducts form at the 2-amino of guanine and the 6-amino of adenine. CE-2,3-Q form only stable adducts, whereas CE-3,4-Q form more than 99% depurinating adducts (7,8).

Clinical evidence supports the hypothesis that CE can cause cancer since women with elevated levels of CE have increased risk for breast cancer (10,11). Moreover, hamsters treated with CE-3,4-Q, which forms primarily depurinating adducts, develop renal tumors following CE-3,4-Q, but not with CE-2,3-Q, which forms only stable adducts (12-14). These results indicate that apurinic sites must play a significant role in the development of mutations.

Most of the time apurinic sites and stable adducts are repaired by endogenous repair systems. Apurinic sites are usually repaired by the base excision method (15), whereas stable adducts are repaired by nucleotide excision repair (16,17). Sometimes, however, this damage is not repaired or is misrepaired and this results in a mutation that may lead to cancer. In the case of breast cancer, I think endogenous CE metabolites cause DNA damage that, at times, leads to cancer (18).

BODY

Materials and Methods

Materials: All chemicals were obtained from (Sigma, St. Louis, MO) unless otherwise noted and all enzymes were obtained from Gibco-BRL(Grand Island, NY) unless otherwise noted.

CE-Q Preparation:
CE were prepared as described in (5). CE-Q was synthesized to make a 100mM solution. To do this, 4-OHE_2 (5.62mg) was dissolved in 1 mL acetonitrile and oxidized by manganese(IV) oxide for 10 min. at 0 ^{0}C with constant stirring. 2-OHE_2 was dissolved in acetonitrile and oxidized similarly, but stirred for 15 min. at -40 ^{0} C. This mixture was filtered (22μm filter) and added directly to the DNA solution of interest.

Unscheduled DNA Synthesis:
ACI rat mammary glands were excised and placed in culture medium (DMEM/F-12,Gibco-BRL, Grand Island, NY) with prolactin (1mg/mL, Sigma, St. Louis, MO), insulin (1mg/mL, Sigma, St. Louis, MO), aldosterone (0.002mg/mL, Sigma, St. Louis, MO), hydrocortisone (0.01mg/mL, Sigma, St. Louis, MO) and penicillin-streptomycin (10,000U/mL, Gibco-BRL, Grand Island, NY). Two glands were floated on mesh per 100 mm dish and were incubated 48 h at 37 ^{0}C in 95% air/5% CO_2 atmosphere (19,20). Then CE-3,4-Q (100 μM) or CE-2,3-Q (100 μM) in acetonitrile in the presence of 100 μCi/dish [^{3}H]thymidine (Amersham, Arlington Heights, IL) was added to the glands in culture. These treated glands, as well as an untreated control and a set treated with DMBA (80μM), were incubated for 24 hours. After this time, the glands were rinsed with unlabeled medium, fixed in 10% formalin for 48 h, paraffinized and cut for slides. The first and third slices were deparaffinized and dipped in KODAK autoradiography Emulsion, type NTB2, and stored at 4^0 C for 6 weeks in the dark. Slides were developed using KODAK developer D-19 and fixed using KODAK Fixer (Rochester, NY). Thymidine grains were tallied and the nucleus to cytoplasm ratios determined (21).

Akaline Agarose Gel Electrophoresis Technique:
MCF-10A1 cells (generous gift from Dr. Judith Christman) were plated at $1.0x10^5$ cells per $60x15mm^2$ dish and allowed to grow overnight in DMEM/F19 without phenol red (Gibco-BRL, Grand Island, NY). The cells were treated with CE-2,3-Q (100 μM) or CE-3,4-Q (100 μM) and incubated at 37 ^{0}C in a 95% air/5% CO_2 atmosphere for time points at 5 min, 15 min, 30 min, 45 min, 1 h, 2 h, 4 h, 6, h, 8 h, and 12 h. At each time point cells were harvested. For early studies this was done by trypsinization and then lysis in 1% SDS, 0.02 M Tris, 0.4 M NaCl, 0.002 M EDTA, pH 8.0, buffer. Later, cells were directly lysed on the dishes using the lysis buffer while remaining on ice the entire time. The resulting solution was extracted with an equal volume of phenol, treated with 0.4 mg proteinase K, incubated at 37 ^{0}C for 1 h and extracted with an equal volume of chloroform. The DNA was precipitated with 2.5 equivalents of ethanol. This DNA was denatured using 1.5 M NaOH and electrophoresed in a 0.25% agarose (Gibco-BRL, Grand Island, NY) gel that was soaked in 30 mM NaOH, 2 mM EDTA for 24 hours prior to running and

run with a 30 mM NaOH, 2 mM EDTA running buffer (22,23).

Electrophoresis Technique Based on Maxam-Gilbert Chemical Sequencing:
pBR322 (Gibco-BRL, Grand Island, NY) was incubated with the restriction enzyme Nhe I overnight at 37 ^{0}C. The resulting product was treated with 0.04 mg proteinase K for 1 h at 37^{0}C. It was then extracted with one equivalent of phenol:chloroform (1:1) and the DNA was precipitated by ethanol. The DNA was then incubated with 0.002 U calf intestine alkaline phosphatase (CIAP) and 10xCIAP dephosphorylation buffer (500 mM Tris-HCl pH 8.5, 1M EDTA) for 30 min at 37 ^{0}C. An additional 0.002 U CIAP was added and incubation was continued for another 30 min. The reaction was stopped using 20 mM Tris, pH7.5, 0.1 μM EDTA, pH8.0, 0.198 mM NaCl, 0.005% SDS. The mixture was extracted with one equivalent of phenol: chloroform (1:1) and ethanol precipitated. The remaining DNA was end labeled using [^{32}P]γ-ATP (10 μCi/μL, Amersham), 5x forward buffer (350 M Tris, pH7.6, 500 mM KCl,, 5mM 2-mercaptoethanol, 50 mM $MgCl_2$), and 2 U T_4 kinase, incubated for 30 min at 37 ^{0}C. The DNA was extracted and precipitated and the resulting DNA was then digested overnight at 37 ^{0}C by EcoRI. This product was electrophoresed in a 1% low-melting point agarose gel and a 231 bp fragment was extracted using 3 U gelase (Epicentre Technologies, Madison, WI). The solution was then phenol: chloroform extracted and the DNA precipitated. The 231bp fragment was redissolved and treated with CE-2,3-Q (100 μM) or CE-3,4-Q (100 μM) for 2 h at 37 ^{0}C. It was then treated with 1 M pipperidine by heating at 95 ^{0}C for 30 min. Parallel samples were prepared using Maxam-Gilbert chemical sequencing techniques (24). These samples were electrophoresed in 5%,6%, 12% or 20% polyacrylamide, 8 M urea denaturing sequencing gels for 3 h at 75 Watts. The resulting bands on the gel were developed by PhosphorImaging (Molecular Dynamics PhosphorImager).

S1 Nuclease Treatment
Double stranded plasmids, pCMV-CAT (kind gift of Dr. Angie Rizzino) were treated with CE-2,3-Q (100 μM) or CE-3,4-Q (100 μM) for 2 h at 37 ^{0}C. They were extracted with an equivalent of phenol:chloroform (1:1) and ethanol precipitated. These plasmids were then treated with 75 U S1 nuclease (Gibco-BRL, Grand Island, NY) for 30 min on ice in 10xS1 buffer (300 mM Na acetate, pH 4.6, 10 mM Zn acetate, 50% (v/v) glycerol). Plasmids were then extracted with one equivalent phenol: chloroform (1:1) and ethanol precipitated. The resulting DNA was electrophoresed in an 0.8% agarose gel or a 15% or 20% polyacrylamide native polyacrylamide gel. The gels were then stained with (0.5 μg/mL) ethidium bromide for 20 min, destained with water for 10 min, if necessary and photographed under UV (25,26).

18mer Oligonucleotides
18 nucleobase oligonucleotides were created containing only a single site for CE-Q binding: CT9G and CT9A. In addition 18mers complementary to these oligos were created (GA10C and GA10T), as well as one containing a deoxyuracil at the 10 position (GA10dU). Oligo sequences are listed in Table 1. All oligos were treated with 0.002 U calf intestine alkaline phosphatase (CIAP, Gibco-BRL, Grand Island, NY) in the presence of 10xCIAP dephosphorylation buffer (500 mM Tris-HCl, pH8.5, 1 mM EDTA) for 30 min at 37 ^{0}C and then 0.002 additional U of

CIAP were added for an additional 30 min of incubation. The reaction was stopped by using CIAP stop solution (20 mM Tris, pH7.5, 0.1 μM EDTA, pH8.0, 0.198 mM NaCl, 0.005% SDS). The oligos were extracted with one equivalent phenol:chloroform (1:1) and ethanol precipitated. The oligos were then radiolabeled using 40 μCi [^{32}P]γ-ATP and 20 U T_4 kinase in 5x forward buffer (350 mM Tris, pH7.6, 500 mM KCl, 5 mM 2-mercaptoethanol, 50mM $MgCl_2$) for 30 min at 37 ^{0}C. The CT9A and CT9G were then treated with 10-100 μM CE-3,4-Q for 2 h at 37 ^{0}C. GA10dU was treated with 0.1 U/μL uracil DNA glycosylase (UDG, Gibco-BRL, Grand Island, NY) for 30 min at 37 ^{0}C. It was then extracted with one equivalent phenol:chloroform (1:1) and ethanol precipitated. The resulting oligo was electrophoresed in a 20% polyacrylamide, 8 M urea denaturing sequencing gel at 75 W for 3 h and compared to a radiolabeled dT oligo (Gibco-BRL, Grand Island, NY) size standard. The radiaoactivity in the gel was imaged using a PhosphorImager (Molecular Dynamics).

Results

Studying the repair process for CE-Q-induced damage is necessary to completely understanding the repair process. As a consequence, the following studies approach this question by first demonstrating a general damage repair process and then looking more specifically at this repair process. Before repair can be studied, however, the ability to demonstrate specific damage is essential. Much of the previous year has been dedicated to this endeavor

Unscheduled DNA Synthesis of CE-2,3-Q or CE-3,4-Q-Treated ACI Rat Mammary Glands:

Statement of work technical objective #1 was to determine the amount of unscheduled DNA synthesis in the ACI rat mammary gland. To do this, DNA damage in ACI rat mammary glands treated in culture with 100 μM CE_2-2,3-Q or 100 μM CE_2-3,4-Q was demonstrated by [^{3}H]thymidine incorporation. Comparison of the thymidine grain ratio in the nucleus versus the cytoplasm showed that treated glands had higher levels of incorporation in their myoepithelial cells than untreated glands. (See Table 2 for an example of the data). Examples of slide slices are shown in figure 1.

Specific Analysis of CE-Q- induced Damage:

To address technical object #2, determination of up-regulated repair enzymes in human MCF- 10A1 cells following CE-Q treatment, studies were then undertaken to develop molecular biological methods to visualize CE-Q-induced DNA damage. We thought that visualization of the damage was essential prior to demonstrating repair of that damage. Several techniques were tried to accomplish this, including alkaline agarose gel techniques, an electrophoresis technique based on Maxam-Gilbert sequencing, utilization of S1 nuclease, and the use of 18-base single-stranded oligomers containing a single reactive site for depurination.

In the alkaline agarose gel technique, MCF10A1 cells were treated with E_2-3,4-Q or E_2-2,3-Q and the DNA collected. This DNA was then electrophoresed in a 0.25 % alkaline agarose gel, following denaturation by 1.5 M NaOH. These studies did not yield any interpretable gels.

Following the alkaline agarose gels, an electrophoresis technique based on Maxam-Gilbert chemical sequencing was tried. In this technique, a 231bp region of pBR322 was excised using Nhe I and EcoR I and [^{32}P]radiolabeled. This portion was then treated with either 100 μM E_2-3,4-Q or 100 μM E_2-2,3-Q. In parallel, the same region was sequenced using Maxam-Gilbert chemical sequencing techniques. The images of these gels demonstrated no particular constant bands formed by apurinic sites in the 231bp treated DNA. In general, however, bands at deoxyadenine consistently developed 60% of the time and bands at deoxyguanine 40% of the time. This confirms the previous studies that show CE-Q binding to deoxyadenine and deoxyguanine. An example of a PhosphorImage for this technique can be seen in Figure 2.

Next, a technique incorporating the use of S1 nuclease was tried. Double stranded-plasmids containing CMV-CAT (kind gift from Dr. Angie Rizzino) were treated with CE-3,4-Q and then with S1 nuclease. Following chloroform:phenol extraction and ethanol precipitation, the DNA was electrophoresed in a 0.8% agarose gel or a 15% or 20% native polyacrylamide gel. The DNA fragments were then visualized following ethidium bromide staining. These gels provided no interpretable results.

Because of the necessity of limiting the identification site for the CE-Q, single-stranded oligos were then created containing a single A or G. In addition, an oligo was created containing a deoxyuradine so that an apurinic site could be created using uracil DNA glycosylase. Using these 18-base single-strand oligonucleotides, including one with a uracil-DNA glycosylase-induced apurinic site, we showed that electrophoresis on 20% polyacrylamide denaturing gels can be used to visualize apurinic site formation in these oligos (see Figure 3). This method is currently being used to visualize CE-Q-induced apurinic site formation.

Discussion

CE-Q treated samples demonstrated elevated levels of damage and repair, in addition to a decreased level of replicating cells. This decrease in replicating cells makes sense since it is recognized that cell cycle stalls via the p53 pathway and others following DNA damage (27). Because of the CE-Q-induced DNA damage, the cells in treated glands probably have entered cell cycle arrest and have lower replicating levels. Although there was a slightly higher level of incorporation in the CE-3,4-Q-treated glands, we do not think this difference compared to the CE-2,3-Q is significant because of the low amount of incorporation overall. This may be physiologically significant, however, since apurinic site repair is ordinary by base excision repair, removing a single or only a few bases, and stable adduct repair uses nucleotide excision repair, removing 17-24 bases. As a result, higher incorporation in the CE-3,4-Q-treated glands may indicate a greater amount of damage by depurinating adducts, requiring more extensive repair.

It was hoped that specific portions of the MCF-10A1 DNA would be more sensitive to CE-Q treatment and so be visible on the gels. (MCF-10a1 cells are derived from fibrocystic breast disease and are thought to be a good model for breast tissue.) In the alkaline agarose

breast disease and are thought to be a good model for breast tissue.) In the alkaline agarose experiments, however, the gels show smears. Because the DNA was prepared on ice, this smear is thought not to be a result of endonuclease and/or exonuclease activity, but this cannot be ruled out. On the other hand, the CE-Q treatment may cause damage at any deoxyadenine or deoxyguanine. So, if not enough of the same locations of damage were present, the damage would not make visible bands. In other words, this approach may not be sensitive enough to identify the damage.

In the electrophoresis technique derived from Maxam-Gilbert sequencing, I thought that strand breaks formed at CE-Q-initiated apurinic sites would be visible following DNA electrophoresis in a denaturing 5%, 6%, 12%, or 20% polyacrylamide gel. No sites, however, were particularly active; as a result, no specific bands in the treated DNA lanes stood out. Thus, this was not a sensitive enough assay to demonstrate specific DNA damage that could be used to assay repair levels.

S1 nuclease cleaves single stranded portion of DNA and I attempted to use this characteristic to excise DNA at apurinic sites. The pCMV-CAT plasmid contained a wonderful control in that its ori site contains a stem loop structure that is an S1 nuclease substrate. The hopes were that the S1 treatment would cut the double stranded plasmids at apurinic sites, resulting in double strand breaks visible on the gel. Unfortunately, these experiments yielded no interpretable gels. Again, the lack of a specific site for CE-Q-induced apurinic sites resulted in merely a smear. Moreover, often times not even a smear was visible because of the difficulty of S1 to identify a single base mismatch. Ordinarily, S1 cuts single-stranded DNA, but it is severely checked when the single-stranded character spans only a single mismatch.

Single-stranded oligos containing a single deoxyadenine, deoxyguanine, or deoxyuracil were created to provide a single site at which a CE-Q could bind. Although only one site is available, we continue to have difficulty visualizing CE-3,4-Q-induced apurinic site formation because the level of sensitivity of this technique lies at or slightly above the level of apurinic sites formation. UDG treatment of GA10dU has provided an easily visualized apurinic site. By using dilutions of this treated oligo, the level of sensitivity for this assay is presently being determined.

Statement of Work Discussion

A copy of the original statement of work is included as Appendix 1.

Once the CE-Q-induced damage is visualized, double strand oligos containing apurinic sites will be treated with cellular extracts from MCF-10A1 cells to see whether DNA repair can be initiated and visualized using this gel electrophoresis technique. Perhaps these experiments will be undertaken using GA10dU, which can be coupled to its complementary strand, CT9A, so that double-stranded oligo may be used. These repair assays should, in part, fulfill the goals of statement of work technical objective #4 (scheduled for months 16-36) in which the effects of different catechol estrogen-induced damage on repair enzyme function will be determined. As for technical object #2: determination of up-regulated repair enzymes in human cell culture

(scheduled for months 7-18), it was delayed until a molecular biological method to determine damage and subsequent repair had been derived. Technical objective #2 could be addressed as early as March of next year. In addition a new technique using reporter genes is currently being developed to assay for repair of specifically created damage and may be applicable to addressing technical objectives #2 and #4 by using portions of a technique described in Napolitano and Fuchs (28). As well, I am implementing a different type of apurinic site assay derived from the technique described in Kubo, et al. and Nakamura, et al (29,30).

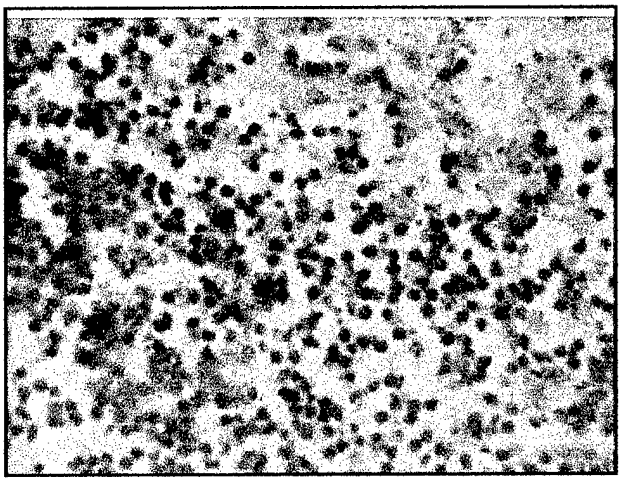
Table 1:

Name of oligo	Sequence (5'—>3')
CT9A	CTT TCC TC**A** TCT TCC CTT
CT9G	CTT TCC TC**G** TCT TCC CTT
GA10dU	AAG GGA AGA **dU**GA GGA AAG
GA10C	AAG GGA AGA **C**GA GGA AAG
GA10T	AAG GGA AGA **T**GA GGA AAG

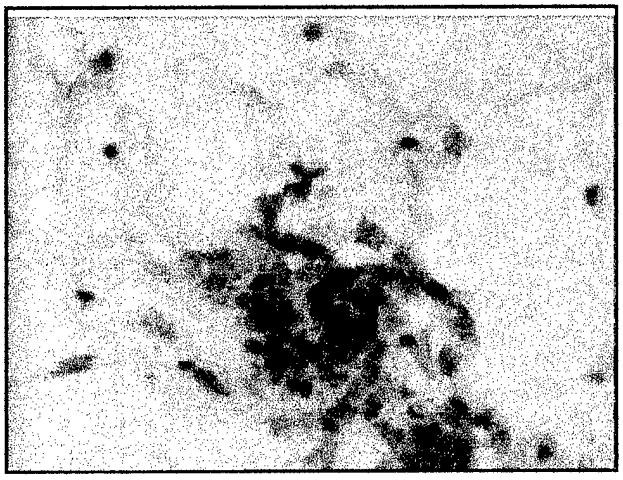
Table 2:

Treatment Method	N/C ± s.d.
100μM CE-2,3,-Q	1.420 ± 0.141
100μM CE-3,4-Q	3.115 ± 0.375
no treatment	0.838 ± 0.100
80μM DMBA	1.603 ± 0.350

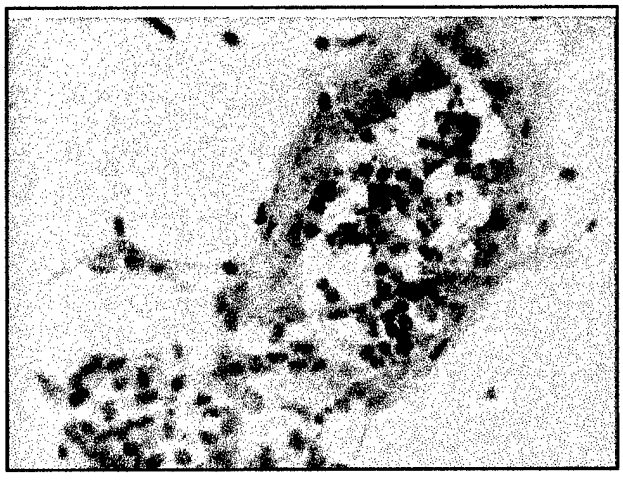
Figure 1. Unscheduled DNA Synthesis



Untreated

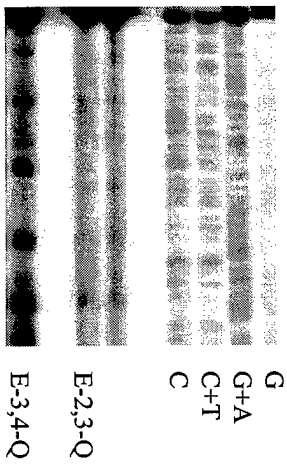


E-2,3-Q



E-3,4-Q

Figure 2. Maxam-Gilbert Gel



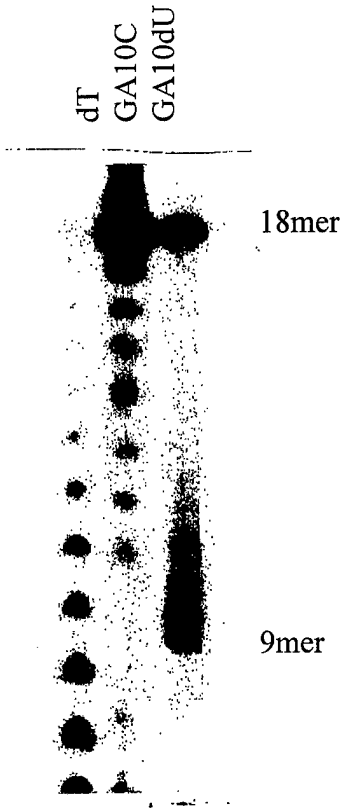


Figure 3. 18mer Oligonucleotide

CONCLUSIONS

In summary, in the past year, I have been able to demonstrate that CE-2,3-Q and CE-3,4-Q can initiate repair processes in ACI rat mammary gland cultures above the level of control treated glands. Although this is no surprise, it was necessary to demonstrate this in an *in vitro/in vivo* system. In addition, I have spent a great deal of time developing a molecular biology- based technique to visualize specific DNA damage so that repair may be more easily quantified. Attempts to use alkaline agarose gels, a Maxam-Gilbert like electrophoresis technique, and electrophoresis of DNA from treated cells were not successful. Using single-stranded, 18-base oligos run in sequencing gels, however, appears to be very promising.

Since a technique has been developed to identify damage, now repair of that damage may be studied using variously damaged double stranded oligos repaired *in vitro* by cellular extracts and damage-containing plasmids repaired *in vivo* in transfected cells (16,31). Understanding the repair or misrepair of DNA damage by CE helps the understanding of breast cancer development to move forward. Perhaps therapies that address this mis-repair may be developed. Prevention of breast cancer by inhibition of CE-induced damage through understanding of the damage/mis-repair/mutation pathway may, however, be a more likely outgrowth of these studies.

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APPENDIX 1

Statement of Work

Technical Objective #1: Unscheduled DNA synthesis in ACI rat mammary gland

Task 1 Months 0-6 Determine optimal levels for treatment with catechol estrogen quinones and optimal amount of [^{3}H]thymidine for our ACI rat mammary gland organ cultures. Determine damaged cell type.

Task 2 Months 6-9 Conduct the experiments with optimal concentrations of catechol estrogen quinones and with optimal levels of [^{3}H]thymidine.

Technical Objective #2: Determination of up-regulated repair enzymes in human cell culture

Task 3 Months 7-8 Establish appropriate cultures of human breast cells that correspond to cell type determined in task 1. Design and obtain oligonucleotides to be used as probes for enzymes that are recognized to play role in repair, i.e. human AP endonuclease, dRpase, 5'->3' exonuclease.

Task 4 Months 8-18 Probe for expected enzymes and determine when they appear during the cell cycle.

Technical Objective #3: Determination of levels of up-regulated repair enzymes in human breast from women with and without breast cancer

Task 5 Months 16-24 Use oligonucleotide probes, designed and used in tasks 3 and 4, to determine levels of up-regulated repair enzymes in human breast specimens.

Technical Objective #4: Determination of effects of different catechol estrogen-induced damages on repair enzyme function

Task 6 Months 16-18 Design and obtain oligonucleotides (18 mers). Establish MCF-7 cultures for repair assay and set up repair assay.

Task 7 Months 18-34 Determine best conditions for repair assay and carry out assay with all of the designed oligonucleotides.

Task 8 Months 28-36 Carry out DNase footprinting to determine location of repair subunits in relation to DNA damages.